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(54) Title: RECOMBINANT THERAPIES FOR INFECTION AND HYPERPROLIFERATIVE DISORDERS

(57) Abstract

Host cells may be treated for an infection or a hyperproliferative disorder which is characterized by the presence, in the affected cells, of a trans-acting factor capable of regulating gene expression by inserting into the cells a polynucleotide construct having a cis-acting regulatory sequence which is regulated by the trans-acting factor and an effector gene which renders said cell susceptible to protection or destruction. For example, the cis-acting region may be homologous to the HIV tar region, and the effector gene may encode ricin A or HSV-1 thymidine kinase. Upon infection with HIV, the HIV tat protein activates the tar region, and induces transcription and expression of ricin A, resulting in cell death, or of HSV-1 tk, resulting in cell death upon treatment with dideoxynucleoside agents such as acyclovir and gancyclovir.

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RECOMBINANT THERAPIES FOR INFECTION AND
HYPERPROLIFERATIVE DISORDERS

Description

15 Related Applications

This application is a continuation-in-part of copending U.S. Patent Application Serial No. 300,637, filed 23 January 1989, incorporated herein by reference in full.

20 Technical Field

This invention relates to genetic engineering and the treatment of hyperproliferative disorders and infection.

Background of the Invention

25 The therapy of viral infection is in its infancy. Bacterial infection is typically treated with agents, such as antibiotics, which take advantage of the differences in metabolism between the infecting organism and its host. However, viruses largely employ the host's own enzymes to
30 effect their replication, and thus leave few opportunities for pharmacological intervention. By employing strong regulatory elements, the virus obtains transcription and translation of its own genes at the expense of host genes.

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In mammals, viral infection is combatted naturally by cytotoxic T-lymphocytes, which recognize viral proteins when expressed on the surface of host cells, and lyse the infected cells. Destruction of the infected cell prevents the further replication of virus. Other defenses include the expression of interferon, which inhibits protein synthesis and viral budding, and expression of antibodies, which remove free viral particles from body fluids. However, induction of these natural mechanisms require exposure of the viral proteins to the immune system. Many viruses, for example herpes simplex virus-1 (HSV-1), exhibit a dormant or latent phase, during which little or no protein synthesis is conducted. The viral infection is essentially invisible to the immune system during such phases.

Retroviruses carry the infectious form of their genome in the form of a strand of RNA. Upon infection, the RNA genome is reverse-transcribed into DNA, and is typically then integrated into the host's chromosomal DNA at a random site. On occasion, integration occurs at a site which truncates a gene encoding an essential cellular receptor or growth factor, or which places such a gene under control of the strong viral cis-acting regulatory element, which may result in transformation of the cell into a malignant state.

Viruses may also be oncogenic due to the action of their trans-acting regulatory factors on host cell regulatory sequences. In fact, oncogenesis was the characteristic which lead to the discovery of the first known retroviruses to infect humans. HTLV-I and HTLV-II (human T-lymphotropic viruses I and II) were identified in the blood cells of patients suffering from adult T-cell leukemia (ATL), and are believed to induce neoplastic transformation by the action of their trans activating factors on lymphocyte promoter

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regions. HTLV-I and II preferentially infect human lymphocytes, and on occasion, cause their transformation into malignancy. Since then, two additional retroviruses have been found to infect humans: HIV-I and HIV-II, the etiological agents of AIDS. However, HIV-I and II apparently contribute to cancer only through their immunosuppressive effects.

HIV I and II apparently infect cells which express the CD4 surface protein. This protein is present in abundance on thymocytes and some T-lymphocytes, and to a lesser extent, on some antigen-presenting cells. HIV infection is initially characterized by flu-like symptoms, followed by a long latency period, which may last five to ten years. Upon entering its active phase, HIV infection results in a rapid decline in the population of "helper" T-lymphocytes (T_H), which is usually recognized as a decline in the ratio of $T4^+/T8^+$ ($CD4^+/CD8^+$) T-lymphocytes. The patient typically experiences severe diarrhea, and if the central nervous system is infected, exhibits a form of dementia. The depletion of T_H cells cripples the immune system, and the patient succumbs to an opportunistic infection by, for example P. carinii or cytomegalovirus, or to Kaposi's sarcoma. The natural immune system appears wholly incapable of combatting HIV infection, despite the typical presence of apparently neutralizing serum antibody titers during latency.

Current therapy for HIV infection per se is limited mainly to administration of AZT to inhibit viral progression, although M.S. Hirsch, J Infect Dis (1988) 157:427-31 reported synergistic inhibition of HIV by AZT with GM-CSF (granulocyte-monocyte colony stimulating factor) or α interferon. AZT (3'-azido-2'-deoxythymidine) is representative of a class of dideoxynucleoside (ddN) antiviral agents. These agents rely on the ability of host DNA polym-

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erases to reject a ddN, and the tendency of viral polymerases to accept ddNs and incorporate them into replicating polynucleotides. Upon incorporation, a ddN stops polymerization, as it lacks the 3' hydroxyl group necessary for the
5 next phosphodiester linkage. The ddNs are typically inactive in their administered form, and depend on phosphorylation by host cell enzymes for conversion to the active triphosphate ddNTP.

H. Mitsuya et al, Nature (1987) 323:773-78 disclosed the organization of the HIV genome, and suggested various strategies for development of HIV therapies. Speculated therapies included administration of antisense RNA (as free strands or encoded by an "antivirus") to inhibit viral transcription, administration of glycosylation
15 inhibitors, administration of interferons to inhibit viral budding, and administration of dideoxynucleoside analogues to inhibit viral replication. Dideoxynucleoside analog drugs useful for HIV therapy (e.g., AZT, ddC, etc.) depend on host cell enzymes for activation by phosphorylation. D.
20 Baltimore, Nature (1988) 335:395-96 suggested treating AIDS by removing bone marrow cells from an infected subject, and transfecting hematopoietic cells in the bone marrow extract with DNA or virus encoding an RNA or protein able to interfere with HIV growth. The DNA could encode RNA that might
25 bind to HIV regulatory proteins, antisense RNA, mutant viral polypeptides, or a viral DNA-binding protein lacking its regulator function. The transfected cells would then be re-introduced into the subject, and provided with a selective advantage to insure dissemination.

30 A.I. Dayton et al, Cell (1986) 44:941-47 disclosed that the HIV tat gene is essential for viral protein synthesis and replication. Dayton suggested that one might inhibit HIV by interference with tat, without otherwise

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affecting the host cell. M.A. Muesing et al, Cell (1987) 48:691-701 disclosed that the tat protein (rather than tat mRNA alone) is essential for transactivation. Muesing also found that a tat-art fusion (having 114 amino acids fused to the C-terminus of tat by deletion of 7 nucleotides) retained full tat activity. A.D. Frankel et al, Science (1988) 240:70-73 disclosed that tat forms metal-linked dimers in vitro, and suggested that possible treatments for AIDS may involve chelation of metal ions, or competition for tat monomer binding. A.D. Frankel et al, Proc Nat Acad Sci USA (1988) 85:6297-30 disclosed the synthesis of an HIV-1 tat fragment which retains the metal-binding properties of tat. The fragment formed heterodimers with native tat, and can displace tat in homodimers. Frankel suggested using the tat fragment to inhibit tat dimerization, using liposomes to deliver the peptides or a tat-fragment gene. The amino acid sequence reported for tat from one HIV-1 isolate is

Met Glu Pro Val Asp Pro Arg Leu Glu Pro
Trp Lys His Pro Gly Ser Gln Pro Lys Thr
20 Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys
Cys Phe His Cys Gln Val Cys Phe Ile Thr
Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys
Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln
Gly Ser Gln Thr His Gln Val Ser Leu Ser
25 Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp
Pro Thr Gly Pro Lys Glu.

A similar protein is found in HIV-2.

The tar sequence, which acts in cis and is regulated by HIV tat, is found approximately at nucleotides +19 to +82 in the HIV-1 genome. The sequence contains two extended, inverted repeats, and is thus predicted to be able to form stem loop structures (Muessing, supra). HIV-2 exhibits a similar region.

35 Haseltine, US 4,738,922 disclosed the HTLV I and II LTR promoter regions, and their use with trans-activators

(luk) in vectors for amplified expression of heterologous proteins, exemplified by chloramphenicol acetyltransferase (CAT). HTLV-I LTR apparently promotes constitutive expression, which is further amplified in the presence of HTLV-I
5 luk, while HTLV-II LTR apparently requires HTLV-II luk for expression. Haseltine mentioned that viral trans-acting factors can alter the expression of host cell genes as well as viral genes, and Haseltine disclosed the concept of inserting a vector having the HTLV LTR fused to a hetero-
10 ologous gene into a cell having the HTLV genome, which results in the trans-activation of the heterologous gene and the overexpression of its product. Haseltine disclosed the use of the HTLV LTR in the absence of luk to generate empty capsid vaccines, and suggested using the HTLV LTR for
15 expression of antigens, to provide for destruction of the cell by monoclonal or polyclonal antibodies.

E.A. Dzierzak et al, Nature (1988) 331:35-41 disclosed a prototypical gene therapy method in mice. Dzierzak removed bone marrow cells from mice, exposed the
20 mice to lethal radiation, and introduced human β -globin (BG) genes into the removed marrow using a retroviral vector, pSV(X)neo. The BG gene was inserted to read in the opposite direction from proviral transcription, and constructs both with and without the viral LTR enhancer regions were pre-
25 pared. The mice were then reconstituted with the recombinant bone marrow, containing hematopoietic stem cells. Dzierzak found that human β -globin was expressed in the resulting recombinant mice, and that the expression was found only in cells of erythroid lineage. J-K Yee et al,
30 Proc Nat Acad Sci USA (1987) 84:5197-201 disclosed construction of retroviral vectors encoding HPRT under control of either the metallothionein promoter or the human cytomegalovirus (hCMV) promoter. Yee found that HPRT expression

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doubled when transcriptional regulatory sequences were deleted from the U3 region of the retroviral LTR.

S.-F. Yu et al, Proc Nat Acad Sci USA (1986) 83:3194-98 disclosed the construction of self-inactivating ("SIN") retroviral gene transfer vectors. SIN vectors are created by deleting the promoter and enhancer sequences from the U3 region of the 3' LTR. A functional U3 region in the 5' LTR permits expression of the recombinant viral genome in appropriate packaging cell lines. However, upon expression of its genomic RNA and reverse transcription into cDNA, the U3 region of the 5' LTR of the original provirus is deleted, and is replaced with the U3 region of the 3' LTR. Thus, when the SIN vector integrates, the non-functional 3' LTR U3 region replaces the functional 5' LTR U3 region, and renders the virus incapable of expressing the full-length genomic transcript. Yu constructed a recombinant virus using the Mo-MuLV LTR regions and packaging (psi) sequence, and inserted a neomycin resistance (Neo) gene under control of either a metallothionein promoter (virus MT-N), an HSV tk promoter (virus TK-N), or a SV40 promoter (virus SV-N) as a selectable marker. Yu also inserted a human c-fos gene under control of a human metallothionein promoter (hMT) into TK-N, and demonstrated inducible transcription of c-fos in NIH 3T3 cells after infection with the recombinant virus.

S.L. Mansour et al, Nature (1988) 336:348-52 disclosed a method for selecting cells after homologous recombination with a linear transfecting vector. A linear vector was prepared having a region homologous to a target gene, a neomycin resistance gene inserted in an exon of the homologous region, and an HSV-tk gene outside the homologous region. Upon specific homologous recombination, the transformed cell displays a phenotype negative for the target region and HSV-tk, and positive for neomycin resistance

(homologous recombination into the target site disrupts the target site gene, and fails to incorporate the tk gene). The phenotype distinguishes cells having homologous recombination from non-specific integration, as the latter cells will display a phenotype positive for the target gene, HSV-tk, and neomycin resistance. Neomycin resistance is tested by culturing cells in neomycin, while tk⁺ is tested by culturing cells in gancyclovir (which is converted to a toxic product by tk).

10 R.D. Palmiter et al, Cell (1987) 80:435-43 disclosed the preparation of transgenic mice having a DNA construct encoding the diphtheria A chain under control of the elastase promoter. The elastase promoter is active only in pancreatic acinar cells, and promotes the expression of
15 elastase. The DNA constructs were microinjected into mouse eggs, and the resulting progeny examined. Transgenic mice in which the construct was active failed to develop normal pancreatic tissue.

M.E. Selsted et al, J Clin Invest (1985) 76:1436-
20 39 disclosed the primary amino acid sequence for three related human cytotoxic effector polypeptides, termed human neutrophil antimicrobial peptides (HNPs). The three HNPs have 29-30 amino acid residues and exhibit activity against bacteria, fungi, and herpes simplex virus (T. Gantz et al, J Clin Invest (1985) 76:1427-35).
25

T.L. Wasmoen et al, J Biol Chem (1988) 263:12509-
63 disclosed the primary amino acid sequence of human eosinophil granule major basic protein (MBP). MBP is an effector polypeptide having 117 amino acid residues, a pI of
30 10.9, and exhibiting cytotoxic activity against mammalian cells and parasites.

M.A. Adam et al, J Virol (1988) 62:3802-06 disclosed retroviral vectors which exhibit efficient RNA pack-

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aging, having a psi⁺ sequence. R.D. Cone et al, Mol Cell Biol (1987) 7:887-97 disclosed the construction of retroviral vectors (pSVX) including a human β -globin gene, and the use of the recombinant vectors to obtain human β -globin expression in a murine erythroleukemia cell line. A.D. Miller et al, Mol Cell Biol (1986) 6:2895-902 disclosed cell lines useful for packaging replication-defective retroviral vectors.

Guild et al, J Virol (1988) 62:3795-801 disclosed retroviral vectors using the Mo-MuLV LTRs and psi (packaging) sequence, useful for transfer of entire genes into mammalian cells. Guild employed β -actin and histone promoters (which are active in essentially all cells) to obtain transcription of neo^r (as a selectable marker). Expression of the neo^r was demonstrated *in vivo*, after virus-infected bone marrow cells were used to reconstitute lethally-irradiated mice.

A.D. Friedman et al, Nature (1988) 335:452-54 disclosed transfection of tk⁻ mouse cells with plasmids expressing HSV-1 tk, and HSV-1 VP16. VP16 acts as a trans activator for transcription of the immediate early genes in herpes simplex virus (HSV-1). On the VP16 plasmid, the promoter was replaced with the Mo-MSV promoter, and the carboxy terminal of the VP16 was deleted. The resulting plasmid codes for a mutant VP16 protein which competes with wild type VP16 for DNA binding. Transfected cells exhibited resistance to HSV-1 replication upon later infection. Friedman suggested that one might induce resistance to HIV by transfecting with a dominant mutant of the HIV trans-activator protein (tat).

J. Sodroski et al, Science (1985) 229:74-77 disclosed the location of the HIV tat gene. J. Sodroski et al, Science (1985) 227:171-73 disclosed construction of a plas-

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mid having CAT under control of the HIV LTR. The HIV LTR contains the transactivating region (tar) gene which is induced by tat. Sodroski discovered that transactivating factors would induce transcription of genes under control of the HIV LTR. B.M. Peterlin et al, Proc Nat Acad Sci USA (1986) 83:9734-38 disclosed plasmids having the HIV tar gene, and the effects of orientation and position of tar on transactivation by tat. Peterlin found that tar functions best when downstream from a promoter and an enhancer. Activation with tat increased transcription to RNA. G.J. Nabel et al, Science (1988) 239:1299-302 disclosed that a HIV tat-III-CAT fusion plasmid could be activated by HSV and adenovirus trans-acting factors.

B.K. Felber et al, Science (1988) 239:184-87 disclosed an assay for HIV, using CD4-expressing cell lines transfected with the chloramphenicol acetyltransferase (CAT) gene under control of the HIV LTR. Upon infection with HIV, the transfected cell lines expressed CAT in proportion to the amount of virus present. Felber suggested using the assay as a means for screening possible anti-HIV drugs for their ability to inhibit viral growth, and for identifying anti-tat drugs.

Disclosure of the Invention

One aspect of the invention is a method for treating host cells for an infection or a hyperproliferative disorder which is characterized by expression of regulatory factors capable of regulating transcription of DNA, by inserting into the cells a polynucleotide construct having a regulatory region which is activated by the regulatory factor, and an effector gene under control of the regulatory region which renders said cell susceptible to protection or destruction. The gene product may destroy the cell dir-

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ectly, as in the case of cytotoxins, or may increase the cell's susceptibility to destruction by pharmacologic agents. Alternatively, the gene product may directly inhibit the infectious or malignant agent, e.g., by competition for binding sites, by binding as antisense RNA, by expression of protein inhibitors or antibodies, by expression of sequence-specific ribozymes, by expression of enzymes which activate anti-viral compounds, and the like. For example, the activation region may be homologous to the HIV tar region, and the effector gene may encode ricin A or HSV-1 thymidine kinase. Upon infection with HIV, the HIV tat protein activates the tar region, and induces transcription and expression of ricin A, resulting in cell death, or of HSV-1 tk, resulting in cytotoxicity when treated with dideoxynucleoside agents such as gancyclovir.

Another aspect of the invention is a DNA construct which accomplishes the method of the invention.

Another aspect of the invention is a composition useful for delivering the DNA constructs of the invention to host cells.

Another aspect of the invention is a method for protecting specific cell populations within an organism from viral infection, by inserting into the cells of the population a polynucleotide construct comprising a cis-acting sequence which promotes expression of a nearby gene only in the presence of trans-acting factors found substantially only in the selected cell population; and under control of the cis-acting sequence, an effector gene which protects the cell or renders it susceptible to protection. Preferably, the cis-acting sequence is derived from the host. The effector gene is preferably a nucleoside kinase, such as HSV-1 thymidine kinase.

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Another aspect of the invention is a polynucleotide construct useful for protecting selected cell populations from infection, which comprises a cis-acting sequence which promotes expression of a nearby gene only in the presence of trans-acting factors found substantially only in the selected cell population; and under control of the cis-acting sequence, an effector gene which protects the cell or renders it susceptible to protection. Preferably, the cis-acting sequence is derived from the host. The effector gene is preferably a nucleoside kinase, such as HSV-1 thymidine kinase.

Brief Description of the Drawings

Figure 1 is a diagram of a generic polynucleotide construct of the invention.

Figure 2 is a diagram of the vector pTB1.

Figure 3 is a diagram of the vector pMXSVNeo-tar/tk.

Figure 4 graphically illustrates the results of the experiment described in Example 5.

Figure 5 graphically illustrates the results of the experiment described in Example 4.

Modes of Carrying Out The Invention

25

A. Definitions

The term "treatment" as used herein refers to reducing or alleviating symptoms in a subject, preventing symptoms from worsening or progressing, inhibition or elimination of the causative agent, or prevention of the infection or disorder in a subject who is free therefrom. Thus, for example, treatment of a cancer patient may be reduction of tumor size, elimination of malignant cells, prevention of

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metastasis, or the prevention of relapse in a patient who has been cured. Treatment of infection includes destruction of the infecting agent, inhibition of or interference with its growth or maturation, neutralization of its pathological effects, and the like.

The term "infection" as used herein includes infection by viruses, bacteria, fungi, and other parasites, such as leishmania and malarial parasites. "Infectious agents" within the scope of this invention include viruses such as HIV-I, HIV-II, HTLV-I, HTLV-II, herpes simplex virus (HSV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human papilloma viruses (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), polio virus, and the like; bacteria such as B. pertussis, and the causative agents of tetanus, diphtheria, cholera, and the like; mycobacteria such as M. tuberculosis, and the causative agent of leprosy; yeasts and fungi such as C. albicans and P. carinii; parasites such as malarial Plasmodia, giardia, and the like. Certain methods and constructs of the invention are most suitable for intracellular infectious agents, such as viruses, malaria and the like, while other methods and constructs are applicable to extracellular infections.

The term "hyperproliferative disorder" refers to disorders characterized by an abnormal or pathological proliferation of cells, for example, cancer, psoriasis, hyperplasia and the like.

The term "cis-acting regulatory sequence" refers to a polynucleotide sequence which is capable of responding to a trans-acting factor, and enhancing transcription of cis-located genes. Most appropriate cis-acting regulatory sequences may be derived from the infectious agent they will be used to combat. For example, the tar region of the HIV-1 LTR is a suitable cis-acting regulatory sequence for treat-

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ment of HIV-1. Where cell-type specific expression is desired, one may employ cis-acting sequences such as, for example, (for T-cells) the CD2 antigen (Genbank HUMATCCD2), IL-2 (Genbank HUMIL2A), IL-2 receptor (Genbank HUMIL2R1),
5 CD1 antigen (Genbank HUMHTA1), CD3 antigen (Genbank HUMATCT31), CD4 antigen (Genbank HUMATCT4), T-cell protease (Genbank MUSSPTCS); for B-cells, IgG (Genbank HUMIGCA1), MHC-1 antigen (Genbank HUMMHA2); for macrophages, Mac-1 antigen (Genbank HUMLAP), IL-1 (Genbank HUMIL1P), and the
10 like. The cis-acting regulatory sequence may be used in multiple tandem copies, in order to increase its competition with the endogenous cis-acting regulatory site. Suitable sequences for treatment of hyperproliferative disorders (especially cancers) are obtainable from the binding sites
15 of known oncoproteins, or by identification of a known protein whose expression is altered by an oncogene. The cis-acting regulatory sequence must be capable of providing sufficient expression of the effector gene to confer susceptibility to protection or destruction of the cell when act-
20 ivated by the trans-acting factor, without allowing substantial constitutive expression in the absence of the trans-acting factor. The choice of cis-acting sequence will depend upon the trans-acting factor associated with the infection or disorder to be treated, and on the effector
25 gene selected. For example, the HIV LTR contains both the tar region, which is highly selective for HIV tat, and also a region activated by the endogenous nuclear factor NF- κ B (the LTR has tandem NF- κ B binding regions). Although the tar sequence strongly suppresses expression in the absence
30 of tat (see for example Muesing, Peterlin, supra), the cis-acting elements upstream of the tar sequence influence the degree of constitutive expression ("leakiness") that occurs in the absence of tat. The degree of leakiness in the

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absence of tat can be controlled by deletion or substitution of the cis-acting elements (e.g., NF- κ B binding sites) within the HIV-1 LTR. In order to use the tar sequence with effector genes such as ricin A (which is effective at very low intracellular concentration), one would remove the NF- κ B binding sites using restriction endonucleases prior to inserting the vector into host cells. In contrast, if the effector gene encoded a product which was not particularly toxic to the host cell (for example, the rpt-1 protein found in resting T-lymphocytes: see Patarca, *supra*), but which requires higher concentration for effective viral inhibition, one must employ a cis-acting sequence which provides for strong expression upon induction, but may allow some low-level constitutive expression.

The phrase "susceptible to protection or destruction" means that upon infection or occasion of a hyperproliferative disorder, the presence of the effector gene within the host cell renders the cell either (a) capable of inhibiting the infectious agent or hyperproliferative condition, or (b) the effector gene kills the host cell, or renders it sensitive to an additional exogenous toxic agent. The additional "toxic agent" does not include the host immune system or antibodies, as immunity is often suppressed or ineffective in cases of infection or hyperproliferative diseases. Inhibition of infection may be accomplished by reducing intracellular levels of nutrients or metabolites needed by the infectious agent (e.g., purine or pyrimidine nucleotides, carbohydrates, phosphates, etc.), down-regulating host enzymes required by the infectious agent (for example ribosomal enzymes, endogenous proteases, protein folding enzymes, transport proteins, and the like), down-regulating host cell regulatory factors employed by the infectious agent (for example, the NF- κ B nuclear factor

found in activated lymphocytes which up-regulates HIV-1 transcription), up-regulating viral or host cell factors which suppress viral gene expression, by locking the host cell in a static phase of the mitotic cycle (in the case of certain viruses and hyperproliferative disorders), and the like.

Inhibition may also be accomplished by interference with the infectious agent life cycle, for example by expressing factors which bind to important infectious agent regulatory factors and cause inhibition or deactivation, by expressing host or infectious agent regulatory factors which down-regulate the agent's expression (for example, viruses having a latent phase must have factors which prevent constitutive expression due to their strong regulatory regions), by expressing non-infectious defective mutants of the coat, envelope, or capsid proteins of the infectious agent, by encoding multiple copies of the polynucleotide binding sequence (such that the sequences compete for binding with the infectious agent regulatory agent, and thus limit transcription of the agent), by expression of factors which attack infectious agent proteins, lipids or carbohydrates, (such as neutrophil antimicrobial peptides, eosinophil granule major basic protein, and the like) or which inhibit or prevent processing by infectious agent enzymes. Alternatively, one can encode cytokines which may interfere with or inhibit infectious agents, or which may be cytotoxic, for example, interferons, interleukins, tumor necrosis factors, colony-stimulating factors, transforming growth factors (α and β), epidermal growth factors, and the like. Cytokine expression is also useful in the treatment of hyperproliferative disorders. For example, cytokines may inhibit or kill tumors directly, or may induce differentiation into a final (non-neoplastic) state. Hyperprolifer-

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active disorders may also be treated using any of the other methods noted above which are appropriate. For example, inhibition of cell functions (such as mitotic cycle, protein expression, and the like) can inhibit proliferation.

- 5 Cytotoxic techniques may involve the direct expression of a cellular toxin, such as ricin A, diphtheria toxin, and the like, or may employ one of the above-noted methods to a degree which results in cell death (for example, complete inhibition of cellular respiration).
- 10 Alternatively, one may express an enzyme or protein which renders the cell susceptible to an additional agent, for example, elevated expression of a nucleoside kinase may render the host cell susceptible to the action of gancyclovir or acyclovir, or may increase the potency of an anti-
- 15 viral agent such as AZT. Dideoxynucleoside analogues (ddNs) such as AZT, dideoxycytidine, acyclovir, gancyclovir, and the like, are relatively non-toxic in their non-phosphorylated form. However, specific viral or intracellular enzymes may convert the ddNs to the corresponding triphosphates, at
- 20 which point the agent acts as a chain termination agent during transcription or replication. The utility of ddNs is based on the fact that (1) viral polymerases exhibit a higher affinity for ddNs than mammalian polymerases, and (2) some viral nucleoside kinases exhibit a higher rate of phosphorylation for ddNs than mammalian kinases. Thus, viral
- 25 enzymes (and consequently viral genes) are more affected by chain termination than are mammalian enzymes and genes. The HSV-1 thymidine kinase (HSV-1 tk) is more efficient at converting ddNs to the corresponding triphosphates, thus rendering cells having active HSV-1 tk more susceptible to
- 30 ddNs.

The term "effector gene" refers to a polynucleotide sequence which, when expressed (due to action by the

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trans-acting factor on the cis-acting regulatory sequence), renders the host cell susceptible to protection or destruction, as defined above. Cytotoxic proteins within the scope of this invention do not include possibly toxic viral proteins under control of its normal viral regulatory region. The effector gene must be a gene which is not naturally regulated by the cis-acting regulatory region employed. One class of suitable effector genes includes genes encoding a nucleoside kinase capable of phosphorylating dideoxynucleoside analogues at a greater rate than host cell nucleoside kinases, for example HSV-1 thymidine kinase, guanine kinase, plant nucleoside phosphotransferase, Leishmania donovani purine 2'-deoxyribonucleosidease, L. donovani hypoxanthine-guanine phosphoribosyltransferase, and other suitable enzymes of nucleoside metabolism capable of activating nucleoside antiviral or chemotherapeutic agents. Another class of effector genes includes genes functional at the mRNA level, such as antisense mRNA, and ribozymes (V. Walbot et al, Nature (1988) 334:196-97). Another class of effector genes includes genes encoding cytokines useful for antiviral or anti-hyperproliferative disorders, such as tumor necrosis factor, α interferon, β interferon, gamma interferon, transforming growth factor- β , inhibitory peptides, and interleukin-2. Other effector genes within this invention include protease inhibitors, which may inhibit essential protein processing, and inhibitors of glycosylation, phosphorylation, or myristylation of viral proteins. RNase is also suitable. One may alternatively "titrate" the trans-acting factor by providing a plurality of cis-acting sequences in conjunction with a strong termination sequence.

In the context of cell-specific expression, the effector gene must confer susceptibility of the cell to protection, but not destruction. Thus, one avoids deleting an

entire class of cells, such as the class of T_H cells. This technique can be employed to increase the therapeutic ratio of certain antiviral and chemotherapeutic agents, such as AZT. For example, AZT inhibits HIV replication, but is relatively toxic to bone marrow cells. T-lymphocytes are commonly infected in HIV infection, and are more tolerant of AZT. By transfecting a bone marrow aspirate with a polynucleotide construct which expresses, for example, HSV-1 tk only in T-lymphocytes (for example, under control of the CD4 antigen promoter), reintroducing the cells into the subject, and administering an amount of AZT less than the normal dosage, one can increase the intracellular concentration of phosphorylated AZT in tolerant T-lymphocytes, without increasing the phosphorylated AZT levels in sensitive stem cells (in bone marrow).

Polynucleotide constructs of the invention may also be designed to treat autoimmune disorders, by using a cis-acting sequence which responds to a trans-acting factor characteristic of those specific immune cells which participate in the particular autoimmune disorder treated, and an effector gene which suppresses the activity of those cells.

The term "antisense mRNA" refers to mRNA which is complementary to and capable of forming dsRNA complexes with a "sense" strand of mRNA. Hybridization of mRNA inhibits its translation into proteins, thus, antisense mRNA acts as a very specific protein synthesis inhibitor. Antisense mRNA may be protective, if it complements for example a viral protein mRNA or mRNA transcribed from an active oncogene. Antisense mRNA may be destructive, for example where it complements an essential host cell enzyme, such as a house-keeping enzyme with no effective alternate pathway, or renders the host cell susceptible to agents which inhibit the

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alternate pathways. See also G. Zon et al, EP 288,163, which disclosed the use of oligodeoxynucleotides for inhibition of retroviral replication and oncogenic proliferation.

The term "ribozyme" refers to a catalytic RNA polynucleotide capable of catalyzing RNA cleavage at a specific sequence. Ribozymes are useful for attacking particular mRNA molecules. For example, in chronic myelogenous leukemia, a chromosomal translocation involving the genes bcr and abl (Philadelphia chromosome) results in expression of a bcr-abl fusion protein, which is believed to result in abnormal function of the abl oncoprotein. Because the fusion between the bcr and abl genes occurs at points within one of two introns, the spliced bcr-abl fusion transcript contains only two possible sequences at the splice junction between the bcr and abl exons. As the bcr-abl mRNA will only occur in lymphoid cells which have undergone this oncogenic chromosome translocation, a ribozyme specific for either of the two bcr-abl fusion mRNA splice junctions may be prepared, and thus may inhibit expression of the corresponding oncoprotein.

The term "vector" as used herein refers to any polynucleotide construct capable of encoding the cis-acting regulatory sequence and the effector gene(s) selected, and capable of transferring these genes into suitable target cells. Vectors may be linear or circular, double stranded or single stranded. Vectors may comprise DNA, RNA, DNA/RNA hybrids, and DNA and/or RNA polynucleotides having chemically modified bases. Suitable vectors within the scope of this invention include linear dsDNA segments, plasmids, recombinant viruses (e.g., recombinant vaccinia, adenovirus, adeno-associated viruses, and the like), replication-defective retroviral vectors (including human, simian, murine, and the like), suitably non-pathogenic derivatives

of replication-competent retroviral vectors, and the like. Replication defective retroviral vectors are presently preferred.

5 B. General Method

Preparation of polynucleotide constructs is conducted using methods known generally in the art. Once an infectious agent or hyperproliferative disorder has been selected for treatment, the first step is to identify an
10 associated trans-acting factor, an appropriate cis-acting sequence, and an effector gene.

Some viral trans-acting factors are already known and characterized. Other viral trans-acting factors may be identified by deletion analysis of the cloned viral genome.
15 For example, a new virus may be cloned and sequenced using standard techniques, and the open reading frames identified. Then, deletion mutants are prepared using restriction enzymes, and the mutant viruses assayed for transcriptional competence in suitable host cells. Mutants which exhibit
20 very low mRNA transcription probably lack either a gene encoding a trans-acting factor, or a cis-acting regulatory sequence. Whether the deletion affects the trans-acting factor or the cis-acting sequence can generally be determined by examination of the position and content of the genomic sequence (e.g., cis-acting viral regulatory sequences
25 are generally found upstream of open reading frames). The sequence of the cis-acting viral region may be compared for homology to known cis-acting regions. As homologous cis-acting sequences may react with the same trans-acting factor,
30 appropriate endogenous trans-acting factors may thus be identified. Alternatively, viral proteins may be expressed recombinantly in appropriate hosts (e.g., bacteria, yeast, mammalian cell culture), and purified extracts labeled and

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screened for binding to the viral genome library. In this manner, appropriate trans-acting factor/cis-acting sequence pairs may be identified. The suitability of the pair may be evaluated using the CAT expression assay detailed below and
5 in the examples. In these assays, the cis-acting sequence is cloned into a suitable vector, and a suitable reporter gene (e.g., CAT, β -galactosidase, etc.) ligated to the cis-acting sequence to provide for cis-control. The test construct is then transferred into a suitable host cell (e.g.,
10 mammalian cell culture) and assayed for reporter gene expression in the presence and absence of the trans-acting factor. Suitable effector genes are known in the art, or may be cloned using standard techniques.

Intracellular parasites (including viruses) often
15 induce interferon expression. Thus, isolation of the interferon cis-acting sequence should allow identification of trans-acting factors involved in the anti-parasite response, and preparation of appropriate polynucleotide constructs of the invention.

20 Hyperproliferative disorders are characterized by inappropriate gene regulation or gene product activity, typically involving cell growth or differentiation factors, and occasionally genes encoding structural proteins. Inappropriate gene regulation may result from the expression of a
25 dysfunctional trans-acting factor (e.g., a factor in which truncation or mutation has eliminated inhibition of the factor, or which binds irreversibly, etc.), from the over-expression of a trans-acting factor or receptor (e.g., due to the translocational juxtaposition of a strong promoter),
30 or other defects. In any case, the disorder characteristically exhibits a trans-acting factor which is either different from those found in normal cells, or is present in abnormally large quantities. Trans-acting factors encoded

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by viruses associated with hyperproliferative disorders (e.g., HTLV-I, HTLV-II, the E6 and E7 genes of HPV type 16 and type 18) may also be used to regulate expression of an effector gene, as described herein. Once the affected cis-
5 acting sequence is identified, a suitable effector gene may be selected. As the characteristic trans-acting factors are generally at least somewhat homologous to normal trans-acting factors, the cis-acting sequence is likely to exhibit some regulation by endogenous trans-acting factor in normal
10 cells. Thus, preferred effector genes for treatment of hyperproliferative disorders are those which are relatively non-toxic to the host cell at low levels. However, the activity of the trans-acting factors may be constitutively high in the hyperproliferative cells, whereas the activity may be
15 lower and fluctuating (as during the cell cycle) in normal cells. This difference may be exploited to allow accumulation of high levels of effector gene product in hyperplastic cells.

Similarly, many cell-type specific trans-acting
20 factors and cis-acting regulatory sequences have been discovered and described in the literature. Additional cis-acting regulatory sequences may be determined by the methods outlined above. It should be noted that in some instances, a trans-acting factor associated with a hyperproliferative
25 disorder and its cis-acting regulatory sequence will be identical to normal, cell-type specific trans-acting factors and regulatory sequences. In such cases, the hyperproliferative disorder is typically caused by an excess concentration of trans-acting factor. Accordingly, the effector
30 gene must be carefully selected.

Referring to Figure 1, a generic retroviral vector of the invention is depicted. The vector comprises a 5' retroviral LTR and primer binding site (1), a psi encapsid-

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ation (packaging) signal sequence (2), an optional 3' RNA processing signal sequence (3), an effector gene (4), a 5' cis-acting regulatory sequence (5) including a promoter, optionally a promoter (6) and selectable marker gene (7), and a 3' retroviral LTR and primer binding site (8). Sequences 1-8 constitute the retroviral portion of the vector, while sequence 9 is typically derived from a plasmid, and provides for maintenance of the vector in cell culture. The 5' LTR (1) and 3' LTR (8) provide for reverse transcription and integration of the vector into the host cell genome. Suitable LTRs include those derived from Moloney murine leukemia virus (Mo-MuLV) (Shinnick et al, Nature (1981) 293:543), Harvey murine sarcoma virus (Ha-MSV), (Van Beveran et al, Cell (1981) 27:97), HTLV-I, HTLV-II, HIV-1, and HIV-2. In replication-defective retroviruses, the U3 region of the 3' LTR (8) is inactivated. Psi sequence (2) must be included in order for the vector to be included in viral capsids generating in the packaging cell line, but is otherwise inactive once integrated into the host genome. Suitable psi sequences have been described by Cepko et al, Cell (1984) 37:1053-62; Guild et al, supra; and Kriegler et al, Cell (1984) 38:483. Where the vector is nonretroviral, and is to be inserted by transfection rather than infection, the LTR sequences and psi sequence may be omitted. The effector gene (4) is as described above. In "internal promoter" recombinant retroviral vectors, the effector gene (4) is provided with its own promoter/cis-acting regulatory sequence (5) and terminator sequence/polyadenylation sequence (4) (although the terminator and PA sequences may be derived from the effector gene).

The effector gene may be oriented in either direction, but is usually oriented in the direction opposite to the LTR reading frame. In "enhancer replacement" recombin-

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ant retroviral vectors, the effector gene (4) is oriented in the same direction as the LTR, and is not provided with a separate cis-acting regulatory sequence (5) or terminator sequence/polyadenylation sequence (4). Instead, the cis-
5 acting regulatory sequence is provided within the 3' LTR (8) U3 region, so that the effector gene is controlled by the cis-acting regulatory region only after reverse transcription.

The selectable marker (2), if present, encodes a
10 characteristic which enables cells expressing the marker sequence to survive under conditions selecting for transfected cells. The selectable marker typically encodes an enzyme conferring resistance to an antibiotic, for example chloramphenicol, neomycin (Southern et al, J Mol Appl Gen
15 (1982) 1:327-41), or hygromycin (Gritz et al, Gene (1983) 25:179-88). The selectable marker, when employed, is usually provided with its own promoter (6) which allows expression of the marker gene during selection of transfected/infected cells. Suitable marker gene promoters
20 include the histone promoter, HSV-1 tk promoter, and the metallothionein promoter.

Sequence 2 is a polynucleotide maintenance sequence, which provides for the stable maintenance of the vector within producer cells. Typically, sequence 2 is
25 derived from a plasmid, and provides an origin of replication (such as pBR322 ori, or the yeast 2 μ origin) and (usually) an antibiotic resistance marker. Suitable maintenance sequences include pXf3, pBR322, pUC18, pML, and the like. In the case of linear DNA segments used for targeted
30 integration, the vector may be linearized at a point within sequence 2. The LTR regions 1 and 8 are replaced with sequences homologous to the sequence of desired targeted recombination. Sequence 2 is deleted. Sequence 2 includes

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polynucleotide sequences which provide for maintenance and replication in microbial hosts, and additionally includes a counter-selection marker (which provides for deletion of transfected host cells which undergo non-specific integration).

Development of drug activation systems

As a prototype system for activation of cytotoxic drugs, we chose the thymidine kinase gene from Herpes Simplex Virus type 1 (HSV-1 tk). HSV-1 tk can activate a variety of dideoxynucleoside analogues (ddNs) by conversion of these drugs to the (5') monophosphate species. The ddNMPs can act as competitive inhibitors of cellular nucleotide or nucleoside kinases, causing depletion of cellular nucleotide pools; following conversion to triphosphate species by cellular enzymes, ddNTPs can be incorporated into nascent DNA (and possibly RNA) strands, causing chain termination.

One of the best-characterized ddNs which can be activated by HSV-1 tk is acyclovir (acyclo-G). The safety and efficacy of acyclovir derives primarily from its selective activation by HSV-1 tk: acyclo-G is converted to acyclo-GMP several thousandfold more efficiently by HSV-1 thymidine kinase (tk) than by cellular thymidine kinases (K_m for HSV-1 tk = 0.005X K_m Vero tk; V_{rel} HSV-1 tk = 3,000,000X V_{rel} Vero tk). Cellular enzymes then convert acyclo-GMP to acyclo-GTP, which is incorporated into DNA by HSV-1 DNA polymerase causing termination of viral DNA synthesis. Acyclo-GTP inhibits HSV-1 DNA synthesis at about 1/20 the concentration required for similar inhibition of cellular DNA synthesis (P.A. Furman et al, J Virol (1979) 32:72-77).

Once a cis-acting regulatory sequence has been identified, it is tested for effect in a model system. For example, the cis-acting regulatory sequence may be cloned into a plasmid with a suitable reporter gene, such as CAT or

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β -galactosidase. The plasmid is then transfected into a suitable cell line (e., CHO cells, HeLa, HUT78, and the like). The trans-acting factor may be supplied by selecting a host cell line which expresses the trans-acting factor, or
5 by cotransfecting the host cell line with a plasmid encoding the trans-acting factor under control of a different promoter (either inducible or constitutive). The transfected host cells are then assayed for expression of the reporter gene.

10 The suitability of an effector gene such as HSV-1 tk is assessed by cloning into a suitable plasmid under control of the selected cis-acting regulatory region. The plasmid also preferably contains a selectable marker, allowing one to select those cells which have become genetically
15 transformed by the vector.

General structure of thymidine kinase vectors

The general structure of retroviral vectors which can be used to generate recombinant retroviruses containing an effector gene linked to cis-acting regulatory elements is
20 shown in Figure 1. The basic retroviral vectors for expression of HSV-1 tk are derived from Moloney Murine Leukemia Virus (Mo-MuLV). All of the Mo-MuLV genes (gag, pol and env) have been removed from the vectors in order to generate completely replication-defective viruses. The remaining
25 components are required for expression and packaging of viral RNA, reverse transcription and integration, and transcription of the tk gene (and marker gene, if desired) in the target cells. These components consist of the Mo-MuLV Long Terminal Repeats (LTRs), the plus- and minus-strand
30 primer binding sites, and the RNA encapsidation signal (Psi sequence).

In order to regulate the expression of HSV-1 tk in a cell type-specific manner, a hybrid transcription unit is

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constructed in which the HSV-1 tk coding sequence replaces the coding sequence of a cell type-specific transcription unit.

Expression of the HSV-1 tk gene via these vectors can be accomplished in two ways. The first type of vector uses a separate enhancer/regulatory element to regulate expression of HSV-1 tk. In this type of vector transcription of HSV-1 tk proceeds in the opposite orientation to the plus-sense of the provirus (as shown in Figure 1). The tk gene is provided with its own polyadenylation signal, and, if desired, also can be provided with an intron between the tk coding sequence and polyadenylation signal. Potential transcriptional interference in this first class of vectors can be reduced by removing the enhancer/regulatory element sequences from the 3' LTR of the vector. Because only the U3 sequences of the 3' LTR are transcribed into viral RNA, these sequences form both LTRs in the resultant provirus. This allows integration of the proviral cDNA, but results in loss of the enhancers and regulatory elements from both LTRs of the provirus.

In the second type of vector (enhancer replacement vector), the Mo-MLV LTR provides the enhancer and regulatory element sequences that control expression of the HSV-1 tk gene. The Mo-MLV LTR can drive expression of heterologous genes in a variety of cell types (excluding hematopoietic stem cells), but is most efficient in T-cells. Control of cell type-specific gene expression of HSV-1 tk may be achieved by substitution of specific enhancer/regulatory element sequences for the enhancer/regulatory element sequences within the 3' Mo-MLV LTR. The 5' Mo-MLV LTR is retained in the vector to allow efficient expression of viral RNA in the packaging cell line used to generate infectious virus.

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A selectable marker gene can be incorporated into these vectors 3' to the HSV-1 tk gene in order to allow in vitro selection of transformed cells. Expression of the marker gene is provided by its own regulatory element. In general, this regulatory element would be derived from a moderately active cellular gene that is constitutively expressed in all tissues. Some examples of such regulatory elements would be the cytoplasmic β actin promoter, histone promoters, and promoters for glycolytic enzymes.

10 The second type of vector uses a separate enhancer/regulatory element to regulate expression of HSV-1 tk. In this type of vector, transcription of HSV-1 tk proceeds in the opposite orientation to the plus-sense of the provirus. The tk gene is provided with its own polyadenylation signal, and, if desired, also can be provided with an intron between the tk coding sequence and polyadenylation signal. Potential transcriptional interference in this second class of vectors can be reduced by removing the enhancer/regulatory element sequences from the 3' LTR of the vector. This allows integration of the proviral cDNA, but results in loss of the enhancers and regulatory elements from both LTRs of the provirus.

25 A selectable marker can be provided, as in the first class of vectors, and is transcribed in the opposite direction from HSV-1 tk. Transcription thus initiates in the center of the integrated provirus and proceeds in opposite directions, in a manner analogous to the transcription of the E2/E3 genes of human adenoviruses.

Formulation and Administration

30 The polynucleotide constructs of the invention may be formulated, depending on the form of construct. For example, where the vector is a competent (infectious) virus, it may be formulated in the same manner as live virus vac-

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cines. Such formulations are typically buffered, physiologic saline for injection, or buffered oral formulations, either of which may optionally contain antibiotics. Liposome formulations may be prepared by standard methods, for example by suspending lipids in chloroform, drying the lipids onto the walls of a vessel, and hydrating the lipids with a solution containing the polynucleotide construct. Suitable lipids are known in the art, including phosphatidyl serine, phosphatidyl glycerol, lethicin, and the like. A synthetic lipid particularly useful for polynucleotide transfection is N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride, which is commercially available under the name Lipofectin® (available from BRL, Gaithersburg, MD), and is described by P.L. Felgner et al, Proc Natl Acad Sci USA (1987) 84:7413.

Recombinant retroviral vectors may also be formulated for *in vivo* administration (if replication defective). Vectors which are based on HIV-1 or HIV-2 will demonstrate the same cellular tropisms as the native virus, thus providing an efficient and comprehensive means for targeting the appropriate cell populations *in vivo* when treating HIV-1 or HIV-2 infection. Vectors based on HIV may employ the HIV LTR promoters, *tar* sequence, and the *tat* gene for control of expression of heterologous genes. One may refine the target cell specificity of the vector by an appropriate choice of packaging constructs (e.g., *env* gene). For example, the isolate HIV-1_{mac} (M. Quiroga et al, "Modern Approaches to New Vaccines" (1989, Cold Spring Harbor Laboratories) p. 80) is naturally selective for macrophage infection: thus, by packaging the vector in an HIV-1_{mac}-derived envelope, targeting to the macrophages can be effected. Similarly, packaging the vectors using recombinant envelope genes which incorporate sequences derived

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from envelopes or capsids derived of hepatitis B virus (or HAV, or HCV) to obtain targeted delivery to hepatocytes. By using a packaging cell line expressing envelope proteins derived from HIV-2, one can obtain an effective method for

5 targeting the vectors of the invention for protection of T_H -cells, due to the affinity of gp160TM for $CD4^+$ (Smith et al, Science (1987) 238:1704-06).

The mode of administration of polynucleotide constructs of the invention depends on the nature of the vector. For example, retroviral vectors may be administered by

10 inoculation, parenterally or orally. Where the vector is an infectious recombinant virus, administration may be by parenteral injection, oral or intranasal administration, and the like. Liposome formulations are preferably administered

15 by intranasal spray or intravenous injection. The presently preferred method of administration is by incubating defective retroviral vectors with aspirated autologous bone marrow cells or T lymphocytes ex vivo, followed by reintroduction of the treated cells by standard techniques. Briefly,

20 bone marrow cells are aspirated by techniques known in the art for bone marrow transplants, and are generally aspirated from the pelvis. If desired (particularly in the treatment of leukemia and other hyperplastic disorders), the subject may be treated with chemotherapy or radiotherapy following

25 bone marrow aspiration, in order to reduce the burden of infected or hyperproliferating cells. The aspirate may be screened to remove undesirable material (e.g., tumor cells, bacteria, viral particles, and the like), for example by immunoprecipitation, immunoadsorption, immunoreaction with

30 complement fixation, fluorescence-activated cell sorting (FACS), and the like. Ideally, hematopoietic stem cells are labeled and isolated using stem cell-specific MAbs. The screened aspirate is then transfected or infected with a

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construct of the invention. One method of infection is to maintain the aspirated cells in culture in contact with infectious titers of replication-defective retroviral vectors for a period sufficient to insure efficient inoculation. Growth factors for hematopoietic cells (for example IL-3) may be added during cocultivation (E.A. Dzierzak, *supra*). The calcium phosphate transfection techniques known for murine cells may be used (see for example, E.A. Dzierzak, *supra*; S-F. Yu et al, Proc Nat Acad Sci USA (1986) 83:3194-98). Constructs may also be introduced by electroporation (S. Mansour, *supra*). Alternatively, one may employ a transfection agent such as Lipofectin®. Where the vector includes a marker, the infected cells may be screened or selected, if desired. The infected cells are then reintroduced into the subject using methods employed for autologous bone marrow transplantation, typically by intravenous infusion or injection. One may also transfect or infect lymphocytes either *in vivo* or *ex vivo* using constructs of the invention. Infection using ILV-based constructs is preferably conducted *ex vivo*, as the envelope is inactivated by human complement. Specific subsets of the lymphocyte population may be selected by employing monoclonal antibodies to separate the desired subset, or by other methods known in the art: see for example P.W. Kantoff et al, Proc Nat Acad Sci USA (1986) 83:6563-67.

If desired, one may convert some of the lymphocytes or bone marrow cells to packaging cells, by inserting independent constructs capable of expressing the viral genes encoding gag-pol and env, followed by insertion of a vector of the invention. O. Danos et al, Proc Nat Acad Sci USA (1988) 85:6460-64. When the autologous packaging cells are reintroduced into the subject, continuous expression of replication-defective retrovirus during the cell lifetime

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results in transfer of the therapeutic construct of the invention to a large number of host cells.

C. Examples

5 The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

10

EXAMPLE 1

Recombinant Retroviral Vectors

 Retroviral vectors were constructed using standard techniques for manipulation of recombinant DNA (T. Maniatis et al., Molecular Cloning: A Laboratory Manual (New York, 15 Cold Spring Harbor Laboratory, 1982)). Plasmids used in construction of retroviral vectors were obtained from the following sources: pMX1112SVNeo was a gift from M. McMann (UCSF), SV-N was a gift from E. Gilboa (constructed as described by Yu, *supra*).

20

 Plasmid pMX1112SVNeo is constructed by inserting a Cla-Cla fragment of the SV40 early promoter linked to the neo^r gene into the plasmid pMX1112 (described by A.M.C. Brown et al, "Retroviral Vectors", pp. 189-212, in "DNA Cloning: a practical approach, vol. 3 (D.M. Glover, ed, IRL 25 Press, 1987)). The SV40-neo^r gene is positioned between the psi packaging sequence and the 3' Mo-MuLV LTR region on the plasmid. Plasmid pMXSVNeo18 was prepared from pMX1112SVNeo by removing the EcoRI site upstream of the 5' Mo-MuLV LTR, inserting an EcoRI linker at the XhoI site, and adding the 30 pUC18 polylinker between the new EcoRI site and the HindIII site. Plasmid pTAR1 contains the HIV 5' LTR (including the tar sequence), a gene encoding CAT, and an SV40 polyadenylation signal and t intron, and was constructed by cloning

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the XhoI-NarI fragment of HIV-1 (SF2) (R. Sanchez-Pescador et al, Science (1982) 227:484-92) into pML. A synthetic polylinker was added at the NarI site, and the StuI-BamHI fragment of pSV2CAT (C.M. Gorman et al, Proc Nat Acad Sci USA (1982) 79:6777-81) containing the CAT gene and SV40 RNA processing signals was inserted to provide pTAR1. The pTAR1 was cleaved with Asp718 and XhoI, and the resulting fragment cloned into pMXSVNeo18 to produce pTB1, shown in Figure 2. Plasmid pTAT1 was constructed with the HIV tat gene under control of the SV40 EP, and flanked by the SV40 polyadenylation signal (Peterlin et al, *supra*). Plasmid pRT contains the HSV-1 tk gene, promoter, and RNA processing signals. The plasmid ptar/tk was prepared by cloning the BglII-EcoRI fragment of pRT (containing the tk coding sequence and polyadenylation signal) into pTAR1 in place of the CAT gene. Plasmid pMXSVNeo-tar/tk was constructed by cloning the Asp718-EcoRI portion of ptar/tk into the pMXSVNeo18 polylinker (pMXSVNeo-tar/tk is depicted in Figure 3).

Amphotropic retroviruses capable of infecting human cells were prepared by standard techniques (R. Mann et al, Cell (1983) 33:153-59). Briefly, the amphotropic packaging cell line PA-317 (Miller et al, Mol Cell Biol (1986) 6:2895-902; ATCC CRL 9078) was transfected with plasmid vector using the CaPO_4 coprecipitation technique (R. Graham et al, Virology (1973) 52:456-67). 10 μg of plasmid vector plus 25 μg salmon sperm DNA carrier were applied as a CaPO_4 coprecipitate to 5×10^5 PA-317 cells in a 60 mm tissue culture dish for 8-16 h. The precipitate was removed and the monolayer was rinsed with Dulbecco's phosphate-buffered saline. Fresh medium (Dulbecco's modified Eagles medium, DMEM, supplemented with 10% fetal bovine serum and antibiotics) was applied and the cells were allowed to grow and recover for 2 d. The transfected cells were then trypsin-

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ized and transferred to a 150 mm² T-flask and grown for 10-14 d in medium containing 0.8 mg/mL G418. The resulting G418-resistant colonies were trypsinized and cloned by limiting dilution in 96-well tissue culture plates. Recombinant retrovirus produced by individual clones was characterized by infection of human cell lines (HeLa, ATCC CCL 2; HUT78, ATCC TIB 161) to determine viral titer and generation of the correct proviral structure.

Ecotropic recombinant retroviruses were prepared as described above, except that the ecotropic packaging cell line Psi-2 (R. Mann, supra) was used instead of PA-317.

EXAMPLE 2

Infection of cells with recombinant retroviruses

Cell lines were infected with recombinant retroviruses using standard procedures (R. Mann, supra). Briefly, 5×10^6 cells were plated into 60 mm tissue culture dishes and allowed to grow for 16 h. Cell-free supernatants from packaging cells containing plasmid vectors was applied to the target cells for 6-8 h in the presence of 8 µg/mL polybrene. In the case of viruses carrying the G418-resistance marker, cells were grown, selected, and cloned as described for preparation of packing clones, above. Retroviruses carrying HSV-1 tk, but lacking the G418-resistance marker were titered using tk- cell lines: L-M (tk-) (ATCC CCL 1.3) for ecotropic viruses, 143 B cells (ATCC CRL 8303) for amphotropic viruses. Selection for tk⁺ colonies was performed using HAT medium.

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EXAMPLE 3

Assay For Trans-activation by HIV tat

Plasmids pTAR1, pTB1, and pTB2 (having the HIV LTR region in the opposite orientation from pTB1), encoding CAT
5 under control of the HIV tar cis-acting regulatory sequence, were transfected into HeLa cells. Plasmid pTAT1, expressing the HIV tat trans-acting agent, was transfected into half of the HeLa cells. After 48 hours, the cells were lysed, and ¹⁴C-chloramphenicol was incubated with the lysates. The
10 products were extracted with EtOAc, and analyzed by thin-layer chromatography.

The results indicated that CAT was expressed in HeLa cells which contained both a tar-encoding plasmid and a tat-encoding plasmid, but not in HeLa cells lacking the
15 pTAT1 plasmid. Although the level of constitutive expression (leakiness) varied little between pTAR1, pTB1, and pTB2, CAT expression in response to HIV tat was higher in cells containing pTB1 and pTB2 than in cells containing pTAR1 (lacking the Mo-MuLV LTRs and SV40 enhancer).

20

EXAMPLE 4

Antiviral Cytotoxic Effector Genes

Amphotropic MXSVNeo-tar/tk retrovirus was produced, and used to infect HUT78 cells (ATCC TIB 161).
25 Transformed cells ("HUT-tk cells") were selected using 1 mg/mL G418. The sensitivity of mass-cultured HUT-tk cells to acyclovir was determined by growth in medium containing various concentrations of drug. HUT78 cells containing pMXSVNeo-tar/tk ("HUT-tk cells") were pelleted and exposed
30 to polybrene (2 µg/mL) at 37°C for 30 minutes. Cells were then pelleted and resuspended at 10⁵ cells per mL, and exposed to HIV-1 (SF2) for 1.5 hours at 37°C. The cells were then pelleted and resuspended in RPMI 1640 medium con-

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taining 10% fetal calf serum and antibiotics at 10^4 cells per 50 μ L. Fifty μ L of cell suspension was then distributed to wells of a 24 well plate containing 1 mL of medium with 45, 100, or 200 μ M acyclovir. HIV-SF2 was added (sufficient to provide 3 OD in a p25 gag ELISA after 7 days of incubation), and was allowed to propagate for 7 days, and then assayed by anti-p25gag ELISA. Normal HUT78 cells served as controls for the effect of HSV-1 tk. The results of these experiments are shown in Figure 5.

Although HIV replication in HUT78 cells was not significantly affected by acyclovir, replication was strongly inhibited by acyclovir in HUT-tk cells (about 60% with 100 μ M acyclovir). Cytopathic/cytotoxic effects were apparent in both cell types (data not shown). Although analysis of single cell clones of HUT-tk showed variation in sensitivity to acyclovir, the mass-cultured HUT-tk cells were capable of significantly restricting viral replication.

EXAMPLE 5

Assay for inhibition of HIV replication

HIV-infectable cells transformed to an HSV-1 tk positive phenotype can be used for screening nucleoside analogues for antiviral activity and cellular cytotoxicity (Mitsuya, supra).

HUT78 cells containing pMXSVNeo-tar/tk ("HUT-tk cells") were cloned by limiting dilution and assayed for sensitivity to varying concentrations of acyclovir as a measure of constitutive tk expression. An especially sensitive clone was designated TK.5, was chosen to test the ability of cells expressing high levels of tk to activate AZT and resist HIV infection.

HUT78, mass-cultured HUT-tk (from Example 4), and TK.5 cells were pelleted and exposed to polybrene (2 μ g/mL)

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at 37°C for 30 minutes. Cells were then pelleted and resuspended at 10^5 cells per mL, and exposed to HIV-1 (SF2) for 1.5 hours at 37°C. The cells were then pelleted and resuspended in RPMI 1640 medium containing serum and antibiotics at 10^5 cells per 50 μ L. Fifty μ L of cell suspension was then distributed to individual wells of a 24 well plate containing 1 mL of medium with 0, 1, 5, or 10 μ M AZT. The infected cells were culture for 7 days, and virus replication was then assayed by anti-p25gag ELISA. The results are set forth in Figure 4.

EXAMPLE 6

Construction of specific thymidine kinase vectors

The structure of various retroviral vectors containing HSV-1 tk is shown in Figure 1. The functional characteristics and therapeutic applications of these vectors are described below.

SIN-tar/tk-H4Neo: This vector employs the self-inactivating vector described by Yu, using the HIV tar cis-acting sequence and the HSV-1 tk effector gene, and containing neomycin resistance under control of the H4 histone promoter. This plasmid confers susceptibility to ddNs on cells infected with HIV.

SIN-CD4/tk (-H4Neo): This vector is identical to the SIN-tar/tk-H4Neo vector described above, except that the tar cis-acting sequence is replaced with the cis-acting sequence which promotes expression of CD4 antigen in T_H cells. The histone promoter/neomycin resistance marker is optional. This vector confers susceptibility to ddNs on all $CD4^+$ cells. As the CD4 antigen is currently believed to be important in the mode of entry for HIV, this vector would also be useful in the treatment of HIV infection.

SIN-Mac1/tk (-H4Neo): This vector is like SIN-tar/tk-H4Neo, with the HIV tar cis-acting sequence replaced by the cis-acting sequence regulating Mac1 antigen expression in macrophages. The histone promoter/neomycin resistance marker is optional. This vector confers susceptibility to ddNs on all macrophages, which also serve as host cells to HIV.

SIN-tar/rA-H4Neo: This vector employs the HIV-1 tar cis-acting sequence, controlling the ricin A effector gene, using neo^r as a selectable marker.

SIN-fos/ppt: This vector employs the self-inactivating vector described by Yu, using the fos oncogene cis-acting sequence (R. Treisman Cell (1986) 46:567-74) using a plant phosphotransferase effector gene. This vector, in combination with a ddN, would be useful in the treatment of fos-type malignancies.

SIN-pcna/tnf: This vector uses the cis-acting sequence regulating Proliferating Cell Nuclear Antigen (described by J.E. Selis, Leukemia (1988) 2:561-601) and a tumor necrosis factor effector gene. Although PCNA is expressed in all cells, the expression is transient, and occurs only during cell division. Thus, normal cells infected with the vector would not produce toxic concentrations of TNF, whereas neoplastic hyperproliferating cells which are constantly in cell division would express toxic concentrations.

SIN-acg/ifn: This vector employs the cis-acting regulatory sequence which provides expression of a chorionic gonadotropin (S.E. Goelz, Science (1985) 228:187-90) in combination with a β -interferon effector gene. This vector, like the preceding vector, relies on the fact that acg is infrequently expressed in normal cells, and would be useful in cancer treatment.

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SIN-IgG/Rbz: This vector employs the cis-acting sequence from IgG, along with a ribozyme effector gene specific for the bcl/abl splice site which occurs in chronic myelogenous leukemia (CML). As IgG is expressed in B-cells,
5 cell-type protection against CML is obtained.

SIN-e7/Ld: This vector uses the cis-acting sequence responding to the E7 gene of human papilloma virus type 16 (HPV16) in conjunction with a Leishmania donovani purine 2'-deoxyribonucleosidase gene. As expression of
10 HPV16 E7 gene is associated with carcinoma of the uterine cervix (Phelps et al, Cell (1988) 53:539-47), this vector (in combination with the drug 6-methylpurine 2-deoxyriboside) destroys cells in which the E7 gene is expressed.

HIV-2/tk: This vector is derived from HIV-2 from
15 which all of the internal genes are deleted except for the portion of the gag gene which overlaps the psi (encapsidation) signal and the rev-response element from the env gene. The HSV-1 tk gene is inserted 3' from the psi sequence, and is expressed under control of the HIV-2 LTR.
20 If desired, the HIV-1 tar sequence can be substituted for the HIV-2 tar sequence. This vector may be used to express HSV-1 tk from a genome-length RNA by deleting or altering the HIV gag sequence ATG codons (Guild et al, supra), obtaining expression of the HSV-1 tk gene from its own ATG
25 start codon. Alternatively, one may include the HIV-2 env splice acceptor just 5' of the HSV-1 tk gene, without altering the gag start codons, thus obtaining expression of the HSV-1 tk via a spliced subgenomic mRNA. In either case, expression of HSV-1 tk is dependent upon complementation by
30 tat from either a resident HIV-1 provirus or a superinfecting HIV-1 virus. Expression of tk in combination with administration of ACV or GCV then activates toxic levels of the antiviral agent, thus destroying the infected

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cell. These constructs may be packaged in either HIV-2 envelopes or in HIV-1_{IIIB} envelopes, the latter being useful for targeting and destroying infected macrophages.

HIV-2/TCR-tk: This construct incorporates the T-cell receptor promoter internally to drive expression of HSV-1 tk, and contains the RRE from env and psi signal from the 5' end of the HIV-2 gag gene. The 3' LTR of the vector is modified by inserting the TCR α constant region enhancer sequence (described by Ho et al, Proc Nat Acad Sci USA (1989) 86:6714), according the principle disclosed by J. Overhauser et al, J Virol (1985) 54:133-34 for construction of a glucocorticoid-responsive MoMLV. The HIV-2/TCR-tk vector may be used to infect and genetically transform CD4⁺ cells *in vivo*. As neither the vector nor the T4 cell normally contains tat, no expression of transcripts initiating in the viral LTR occurs in the absence of infection with HIV. Initiation of transcription (expression of tk) occurs from the internal TCR promoter. Expression from this promoter is enhanced by the presence of the TCR α enhancers incorporated into the proviral LTRs. This vector provides constitutive expression of tk in the genetically transformed T_H lymphocytes, which in turn activates an antiviral agent (e.g., ACV, ddC and the like). This permits the use of ACV and related compounds in cells in which they would normally not be effective. It also increases the effectiveness of AZT at low concentrations as shown in Figure 4. The ability to employ a variety of antiviral agents should reduce the possibility of development of drug resistance by HIV. An analogous vector may be prepared by substituting MLV virus components for the HIV sequences used, and employing an MLV amphotropic packaging cell line.

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EXAMPLE 7

In Vivo Administration of Constructs

(A) Packaging cell lines for HIV-based vectors are constructed as follows:

5 The gag-pol coding sequence is isolated from HIV-1 or HIV-2, and is inserted into an expression vector under control of the human cytomegalovirus (CMV) major immediate early (MIE) promoter. A suitable polyadenylation sequence is provided 3' from the gag-pol insertion, for example, the
10 SV40 polyA coding sequence. Both the gag-pol and the env sequences must contain the rev-response element (RRE) present in the env coding region, in order to obtain nuclear export (M.H. Malim et al, Nature (1989) 338:254-57).

 The env sequence (including the RRE), which will
15 determine the ultimate specificity of the finished vector, is obtained from a selected HIV-1, HIV-2 or SIV-1 isolate, and is inserted into a separate expression vector under control of the CMV MIE promoter. In this construction, a β -globin polyA sequence is employed. An intron may be
20 inserted 5' to the env coding sequence, optionally containing a coding sequence for a marker or reporter gene (e.g., neo^r).

 Separate similar vectors are constructed to insert tat cDNA and rev cDNA, under control of the SV40 early promoter. These vectors employ SV40 polyA sequences. By separating the coding sequences for gag-pol, env, tat, and rev,
25 the possibility of recombination to regenerate a functional HIV virus is minimized.

 The expression vectors are then used to construct
30 a packaging cell line, by transfection of suitable cell line, for example NIH-3T3, HeLa, or the like. Upon transfection with a retroviral vector of the invention such as HIV-2/TCR-tk, a protective construct of the invention is

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prepared which is capable of infecting the population of host cells that is infected during *bona fide* HIV infection (mainly T_H cells). When administered in combination with suitable antiviral agents such as AZT, ACV, ddC or the like, 5 this treatment protects infected cells from infectious HIV replication.

(B) Macrophage/monocyte-trophic constructs are prepared following the procedure of part A above, but employing the HIV-1_{gag} env gene to provide env protein. 10 Upon transfection with a vector of the invention such as HIV-2/tk, an ablative construct is provided which is capable of superinfecting and destroying host cells which are reservoirs of HIV and infection, particularly macrophages and monocytes.

15

WHAT IS CLAIMED:

1. A method for treating a host cell ex vivo for a hyperproliferative disorder or infection by an infectious agent, said infection or hyperproliferative disorder characterized by a trans-acting regulatory factor capable of regulating expression of genes, which method comprises:
inserting into said cell a polynucleotide construct comprising a cis-acting regulatory sequence which is controllable by said trans-acting regulatory factor; and
an effector gene under the control of the cis-acting regulatory sequence which renders said cell susceptible to protection or destruction.
2. The method of claim 1, wherein said effector gene renders the cell susceptible to destruction.
3. The method of claim 2, wherein said effector gene encodes an antisense mRNA which binds to host cell mRNA encoding an essential host cell protein and inhibits expression of said protein.
4. The method of claim 1, wherein said effector gene encodes a cytotoxic protein.
5. The method of claim 4, wherein said cytotoxic protein is a ribosomal inhibitory protein.
6. The method of claim 5 wherein said ribosomal inhibitory protein is ricin A, or diphtheria toxin.

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7. The method of claim 1 wherein said effector gene encodes a nucleoside kinase capable of phosphorylating dideoxynucleoside analogues at a greater rate than host cell nucleoside kinases.

5

8. The method of claim 1 wherein said effector gene encodes a gene product which renders the host cell susceptible to protection.

10

9. The method of claim 8 wherein said effector gene product comprises antisense mRNA, which mRNA is capable of inhibiting said infection or hyperproliferative disorder.

15

10. The method of claim 9 wherein said antisense mRNA inhibits the infection or hyperproliferative disorder by binding to RNA encoded by said infectious agent or specific to said hyperproliferative disorder.

20

11. The method of claim 10 wherein said infection comprises HIV-1 or HIV-2 infection.

25

12. The method of claim 8, wherein said effector gene product comprises an antibody specific for an antigen associated with said infection or hyperproliferative disorder.

30

13. The method of claim 8, wherein said effector gene product comprises tumor necrosis factor, α interferon, β interferon, gamma interferon, transforming growth factor- β , inhibitory peptides, interleukin-2, or a combination thereof.

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14. The method of claim 8, wherein said effector gene product comprises a protease inhibitor which inhibits processing of infectious agent proteins or proteins specific to said hyperproliferative disorder.

5

15. The method of claim 8, wherein said effector gene product comprises a protein which inhibits glycosylation or myristylation of infectious agent proteins or proteins specific to said hyperproliferative disorder.

10

16. The method of claim 8, wherein said effector gene product comprises an RNase.

17. The method of claim 1, wherein said cis-acting regulatory sequence is present in at least two tandem copies.

18. The method of claim 1, wherein said effector gene comprises:

20

at least two copies of a polynucleotide sequence homologous to a regulatory element region of said infectious agent or hyperproliferative disorder, and capable of binding a trans-acting factor associated with said infectious agent or said hyperproliferative disorder, where said homologous polynucleotide sequences compete with said infectious agent regulatory element regions or said hyperproliferative disorder regulatory elements for said trans-acting factor; and a strong terminator region.

25

19. The method of claim 18, wherein said infectious agent comprises HIV-I or HIV-II, and said activating agent comprises tat.

30

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20. A polynucleotide construct for treating a host cell for a hyperproliferative disorder or infection by an infectious agent, said infection or hyperproliferative disorder characterized by a trans-activating factor capable
5 of regulating expression of DNA, which construct comprises:

a cis-acting regulatory sequence which is controllable by said trans-acting regulatory factor;

an effector gene under the control of the cis-acting regulatory sequence which renders said cell susceptible to protection or destruction.
10

21. The construct of claim 20, which further comprises;

a polynucleotide maintenance sequence which provides for the stable maintenance of said construct within mammalian cells.
15

22. The construct of claim 21 wherein said maintenance sequence comprises a viral vector.
20

23. The construct of claim 20, wherein said polynucleotide construct comprises a recombinant viral vector selected from vaccinia, HIV-1, HIV-2, adenovirus, or adeno-associated virus.
25

24. The construct of claim 22 wherein said viral vector comprises a replication-defective retroviral vector.

25. The construct of claim 24 wherein said replication-defective retroviral vector further comprises a gp160^{env} glycoprotein derived from HIV-1 or HIV-2.
30

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26. The construct of claim 21 wherein said maintenance sequence comprises a gene target vector.

27. The construct of claim 20, wherein said
5 cis-acting regulatory sequence is capable of responding to HIV tat protein.

28. The construct of claim 27, wherein said
effector gene encodes a cytotoxic protein.
10

29. The construct of claim 28 wherein said
cytotoxic protein comprises ricin A or diphtheria toxin.

30. The construct of claim 21 wherein said
15 effector gene encodes a nucleoside kinase capable of phosphorylating dideoxynucleoside analogues at a greater rate than host cell nucleoside kinases.

31. The construct of claim 30, wherein said
20 nucleoside kinase comprises HSV-1 thymidine kinase.

32. The construct of claim 31, which is
pMXSVNeo-tar/tk.

25 33. A composition for treating a host cell for a hyperproliferative disorder or infection by an infectious agent, which composition comprises:

a polynucleotide construct according to claim 21;
and
30 a pharmaceutically acceptable carrier.

34. The composition of claim 33 wherein said
pharmaceutically acceptable carrier comprises a liposome.

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35. The composition of claim 34 wherein said liposome further comprises antibodies specific for host cells desired to be treated.

5

36. A method for protecting a selected population of cells within an organism from infection or hyperproliferative transformation, which method comprises:

introducing into said cells a polynucleotide
10 construct comprising a cis-acting sequence which promotes expression of an effector gene only in the presence of trans-acting factors found substantially only in the selected cell population; and under control of the cis-acting sequence, a effector gene which protects the cells or
15 renders them susceptible to protection.

37. The method of claim 36 wherein said effector gene comprises a nucleoside kinase.

20 38. The method of claim 37 wherein said nucleoside kinase comprises HSV-1 tk, guanine kinase, or plant nucleoside phosphotransferase.

39. The method of claim 38 which further
25 comprises:
administering to said cells ex vivo an antivirally effective amount of a dideoxynucleoside antiviral agent.

40. The method of claim 39 wherein said cis-
30 acting sequence is active in lymphocytes.

41. The method of claim 40 wherein said antiviral agent is AZT.

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42. A polynucleotide construct for protecting a selected population of cells within an organism from infection or hyperproliferative transformation, which construct
5 comprises:

a cis-acting sequence which promotes expression of an effector gene only in the presence of trans-acting factors found substantially only in the selected cell population; and

10 under control of the cis-acting sequence, a effector gene which protects the cells or renders them susceptible to protection.

43. The construct of claim 42 wherein said
15 effector gene comprises a nucleoside kinase.

44. The construct of claim 43 wherein said nucleoside kinase comprises HSV-1 tk, guanine kinase, or plant nucleoside phosphotransferase.

20

45. The method of claim 44 wherein said cis-acting sequence is active in lymphocytes.

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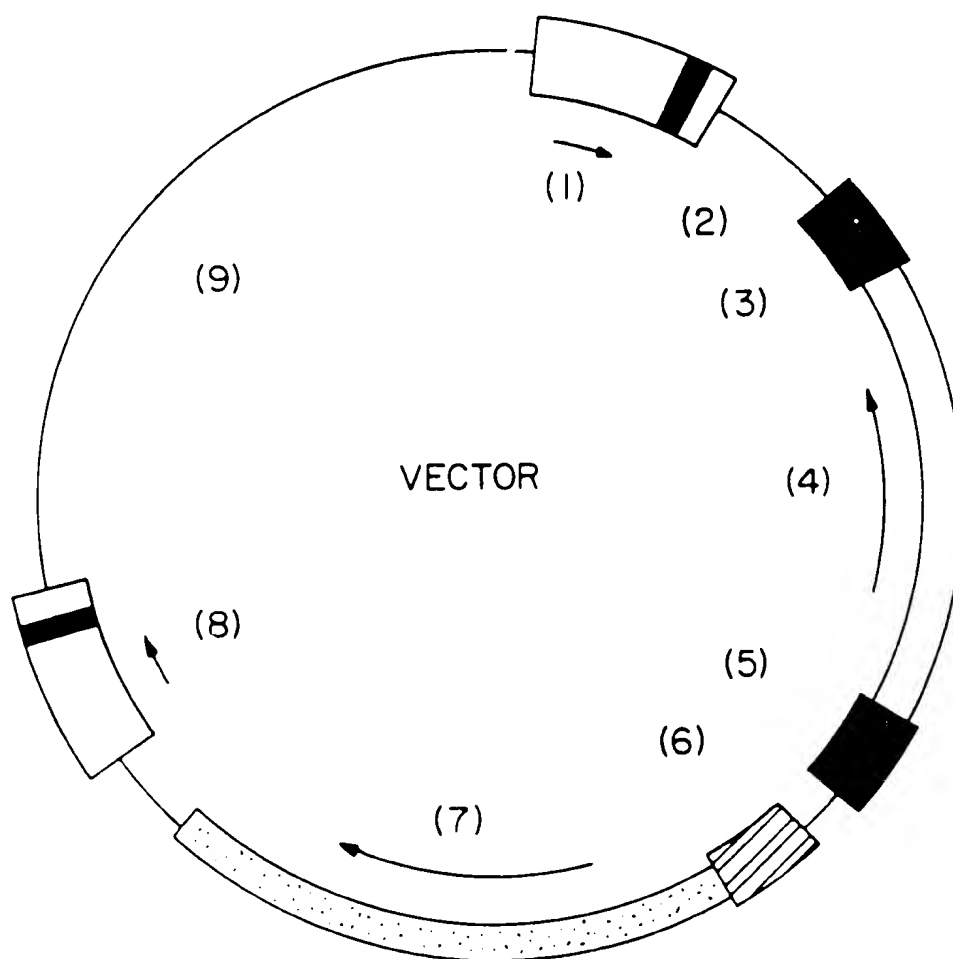


FIG. 1

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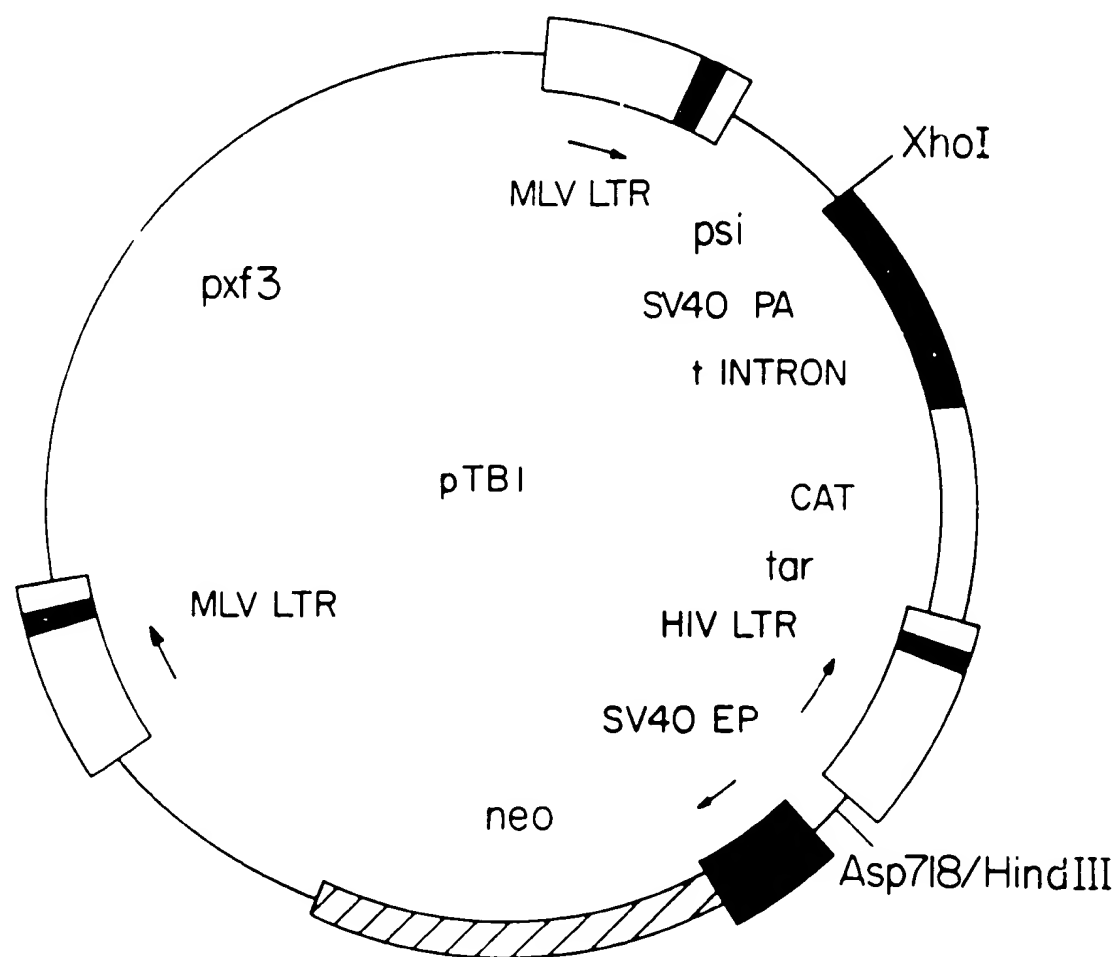


FIG. 2

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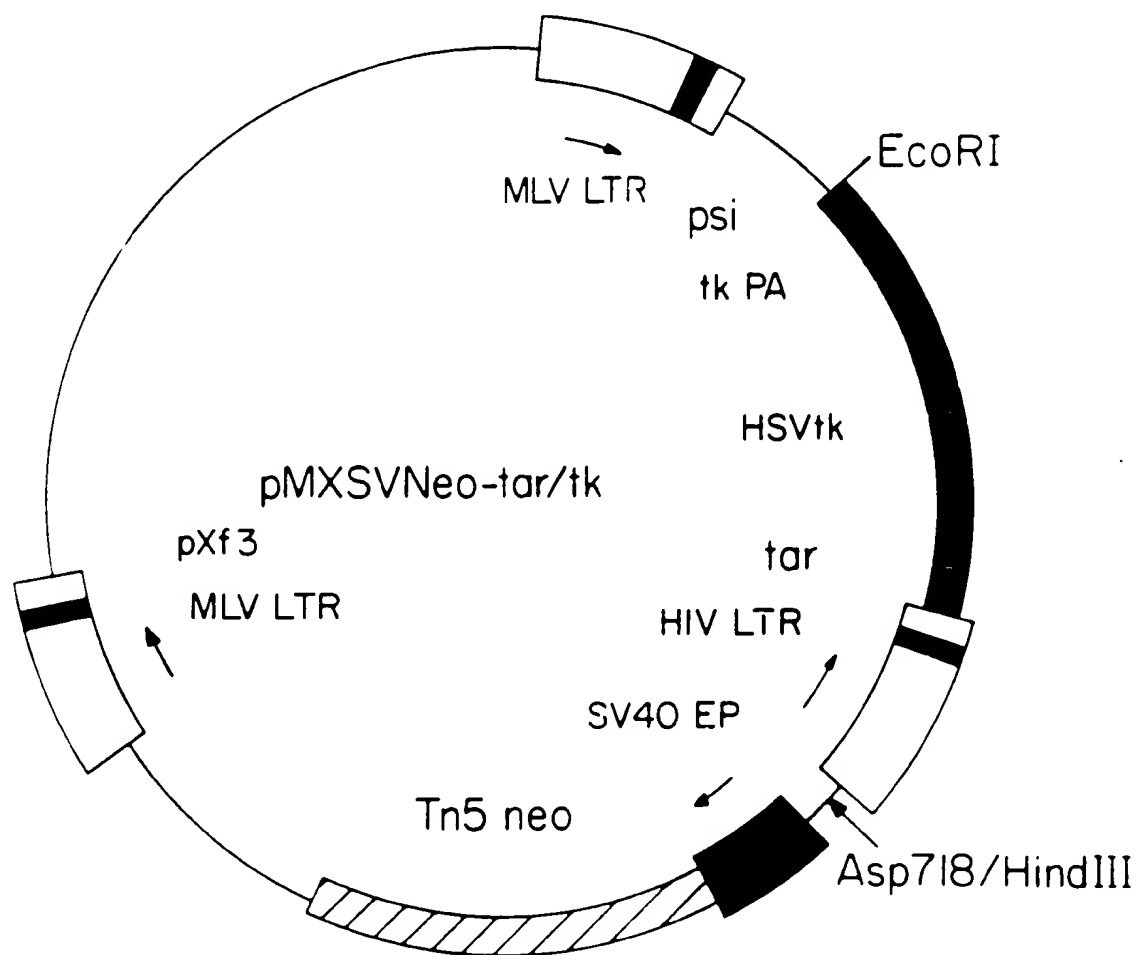


FIG. 3

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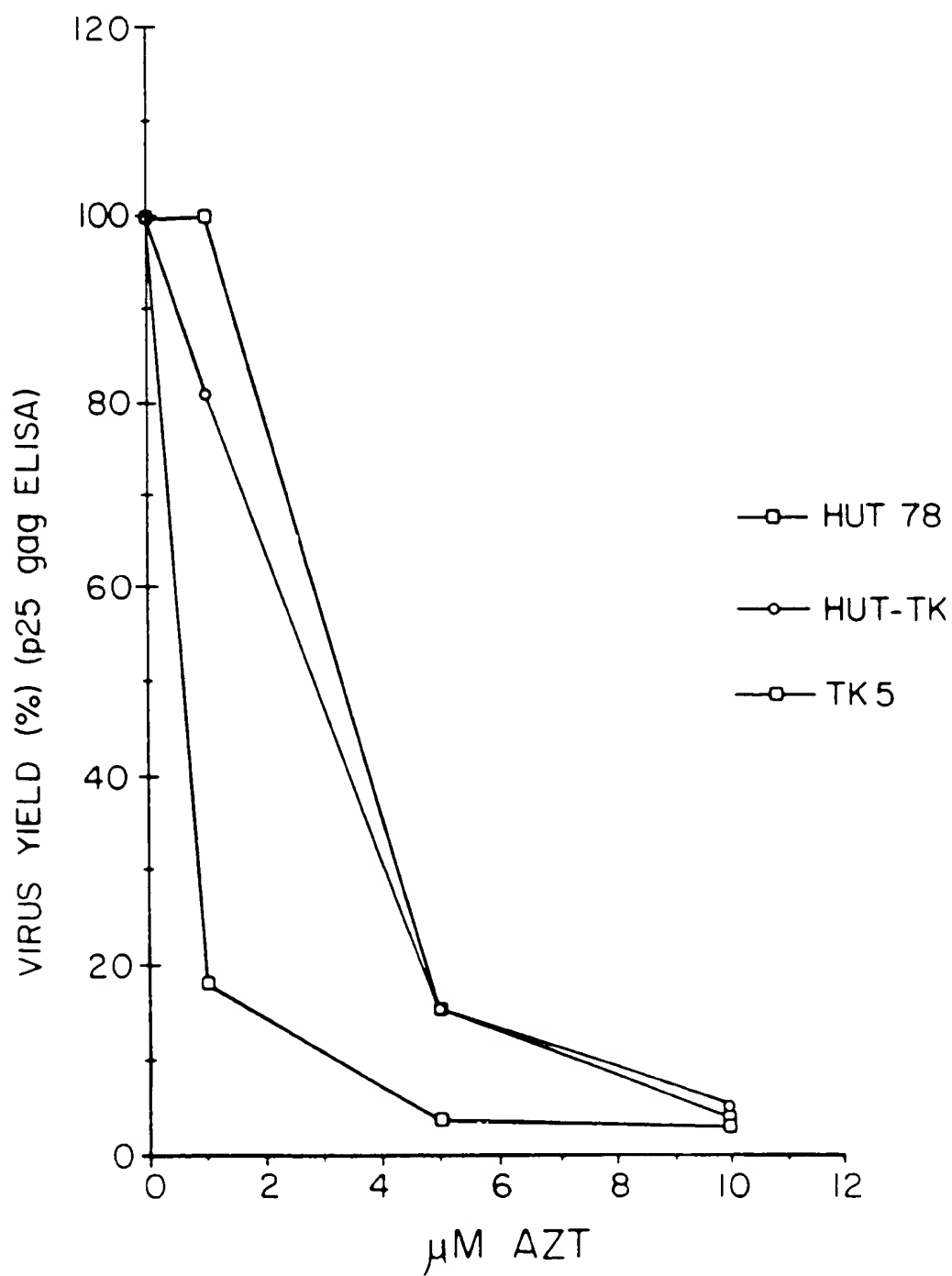


FIG. 4

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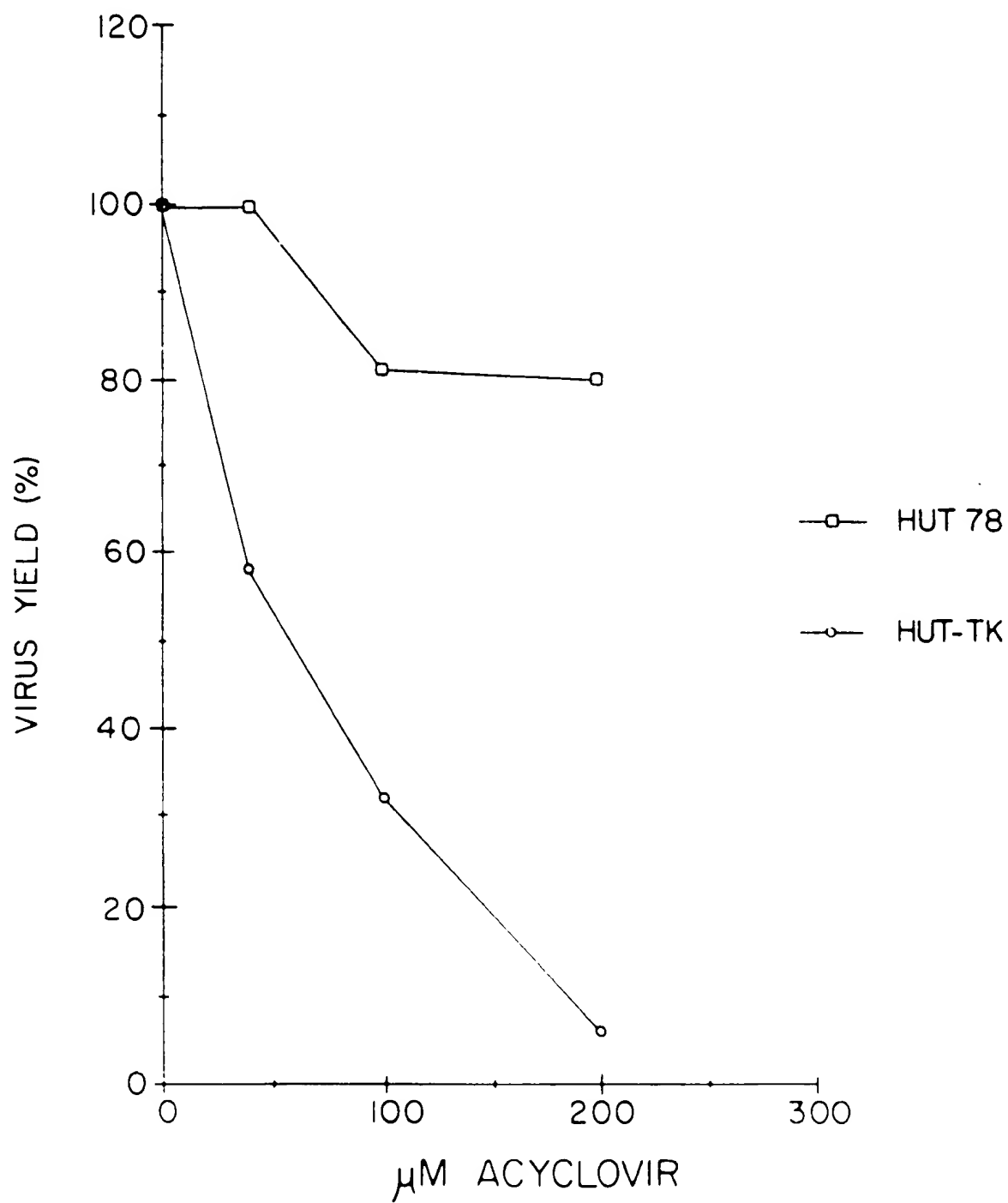


FIG. 5